Optimized loading test to evaluate responsiveness to tetrahydrobiopterin (BH4) in Brazilian patients with phenylalanine hydroxylase deficiency

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Abstract: INTRODUCTION: Recent studies showed that phenylalanine (Phe) plasma concentrations may decrease in some patients with hyperphenylalaninemia (HPA) due to phenylalanine hydroxylase (PAH) deficiency, after the administration of tetrahydrobiopterin (BH(4)). OBJECTIVE: To determine responsiveness to a single dose of BH(4) administered according to an innovative protocol using a combined Phe and BH(4) loading test in Brazilian phenylketonuria (PKU) patients. METHODS: Patient age should be 4 years, and median Phe plasma concentration 600 mol/L when following dietary restrictions. Participants received a simple Phe loading test using 100mg/kg L-Phe (Test 1) and a combined Phe+BH(4) loading test using 100mg/kg L-Phe and 20mg/kg/BH(4) (Test 2). Blood samples were collected at baseline and 3, 11 and 27 h after Phe ingestion (T0, T1, T2 and T3). Responsiveness was defined as: criterion A: plasma Phe reduction of 30% at T1 and T2 for Tests 1 and 2; criterion B: plasma Phe reduction of 30% at T1 and T3 for Tests 1 and 2; and criterion C: at least 30% difference of the areas under the Phe curve for Tests 1 and 2. RESULTS: Eighteen patients (median age 12 yrs; 8 classical PKU; 10 mild PKU) participated in the study. Six patients (2 classical PKU; 4 mild PKU) were classified as responsive according to at least one of the criteria. Responsiveness was concordant when criteria A + B we compared with criterion C (kappa = 0.557; p = 0.017). Of the patients whose genotype was available (n = 16), six had data about BH(4)-responsiveness genotypes described in the literature, which were in agreement with our findings. CONCLUSION: The comparison of simple Phe loading and combined Phe + BH(4) loading seems to be an optimal method to evaluate responsiveness to BH(4) in patients with good metabolic control.

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ABSTRACT

Introduction: Recent studies showed that phenylalanine (Phe) plasma concentrations may decrease in some patients with hyperphenylalaninemia (HPA) due to phenylalanine hydroxylase (PAH) deficiency, after the administration of tetrahydrobiopterin (BH_4).
Objective: To determine responsiveness to a single dose of BH_4 administered according to an innovative protocol using a combined Phe and BH_4 loading test in Brazilian phenylketonuria (PKU) patients.
Methods: Patient age should be ≥4 years, and median Phe plasma concentration ≤600 μmol/L when following dietary restrictions. Participants received a simple Phe loading test using 100 mg/kg L-Phe (Test 1) and a combined Phe + BH_4 loading test using 100 mg/kg L-Phe and 20 mg/kg/BH_4 (Test 2). Blood samples were collected at baseline and 3, 11 and 27 h after Phe ingestion (T0, T1, T2 and T3). Responsiveness was defined as: criterion A: plasma Phe reduction of ≥30% at T1 and T2 for Tests 1 and 2; criterion B: plasma Phe reduction of ≥30% at T1 and T3 for Tests 1 and 2; and criterion C: at least 30% difference of the areas under the Phe curve for Tests 1 and 2.
Results: Eighteen patients (median age 12 yrs; 8 classical PKU; 10 mild PKU) participated in the study. Six patients (2 classical PKU; 4 mild PKU) were classified as responsive according to at least one of the criteria. Responsiveness was concordant when criteria A + B we compared with criterion C (kappa = 0.557; p = 0.017). Of the patients whose genotype was available (n = 16), six had data about BH_4-responsiveness genotypes described in the literature, which were in agreement with our findings.
Conclusion: The comparison of simple Phe loading and combined Phe + BH_4 loading seems to be an optimal method to evaluate responsiveness to BH_4 in patients with good metabolic control.

1. Introduction

Phenylketonuria (PKU) or hyperphenylalaninemia due to phenylalanine hydroxylase (PAH) deficiency is an inborn error of amino acid metabolism characterized by the persistent increase of phenylalanine (Phe) plasma concentration. PAH converts Phe into tyrosine (Tyr), in the presence of its cofactor tetrahydrobiopterin (BH_4) [1].

The standard PKU treatment is based on a Phe-restricted diet and ingestion of a Phe-free amino acid-rich metabolic formula that supplies the daily protein requirements of a patient [1,2]. Because of the toxic effects of high Phe levels, this condition, if left untreated, may lead to neurological impairment, mental retardation and behavioral disorders [3,4].

The necessary dietary restriction and the associated difficulty to adhere to the treatment [4–6], have motivated the search for new PKU management strategies [7]. Since the publication of the study by Kure et al. [8], who described the first case of patients with PKU whose Phe levels decreased after BH_4 administration, several studies

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have been conducted to confirm the efficacy and safety of this medication, which has already been approved by the FDA and the EMEA. Patients are usually evaluated to check their responsiveness and, in case results suggest that they are responsive, that is, that Phe levels will decrease after BH_4 administration, BH_4 supplementation is initiated. Studies, however, have used different protocols to evaluate responsiveness: BH_4 doses are different and may range from 10 to 20 mg/kg/day in a single dose or distributed along the day; test evaluation times range from some hours to weeks or even months; the cut-off point of Phe variation defined to determine responsiveness also varies, and the criterion most frequently adopted is a decrease of 30% in Phe levels 24 h after BH_4 administration. In addition, different diets are used during tests: normal diets, Phe-restricted diet, or even a Phe-loading diet using, for example, powder milk or L-Phe [7,9–22].

This study describes responsiveness to a single dose of BH_4 in a sample of Brazilian patients with PKU and good metabolic control. For that purpose, an innovative protocol with a single Phe plus a combined Phe and BH_4 loading tests was used.

2. Material and methods

This study included patients with PKU seen in the Outpatient Metabolic Disorder Treatment Clinic of the Medical Genetics Service of Hospital de Clínicas de Porto Alegre (ATDM-SGM/HCPA), Porto Alegre, Brazil. At the time this study was conducted, 68 patients with different phenotypes were followed up in the ATDM-SGM/HCPA and 64 of them underwent dietary treatment.

This study was approved by the Ethics in Research Committee of HCPA, and all patients or their guardians signed a written informed consent term.

2.1. Patients

Patients included in the study should be aged ≥4 years and should be under dietary treatment; median Phe plasma levels should be ≤600 μmol/L in the 12 months before the start of the study. The Phe cut-off point adopted ensured that patients with the mild form of the disease, good metabolic control, or both were also included in the test. A previous trial conducted by our study team [23] adopted a different protocol and different inclusion criteria to evaluate responsiveness to BH_4, and most patients had the classical form of the disease and inadequate metabolic control, as their Phe plasma levels had to be ≥360 μmol/L in all measurements during the previous 12 months.

Exclusion criteria were: pregnancy, clinical signs suggestive of liver disease; use of levodopa; allergy to any component of BH_4; median Phe level>600 μmol/L in the measurements made in the 12 months before inclusion in the study; irregular follow-up in the ATDM-SGM/HCPA in the same 12 months; and probable non-compliance with study procedures according to evaluations made by the authors.

PKU types were defined according to Nalin et al. [6], and the patients were classified as having classical PKU or mild PKU.

2.2. Single Phe and combined Phe + BH_4 loading tests

The patients were asked to come to two visits in HCPA and to stay under evaluation for 27 h each time; the two visits were made at a one-week interval.

2.2.1. Simple Phe loading (Test 1)

In the first week, after overnight fasting, blood was collected to measure Phe and Tyr plasma concentrations (T0). After that, patients ingested 100 mg/kg of L-Phe and resumed their usual diet (Phe-restricted diet and supplementation with Phe-free metabolic formula). Blood for Phe and Tyr was then collected at 3 (T1), 11 (T2) and 27 h (T3) after Phe loading.

2.2.2. Combined Phe and BH_4 loading (Test 2)

In the second week, evaluation was conducted using the protocol described by Blau et al. [24], with a modification, as Phe and Tyr levels were not analyzed 7 h after Phe loading. The initial phases of Test 2 (collection at T0, Phe loading, food ingestion, and blood collection at T1) were similar to those described for Test 1. In addition, immediately after collection at T1 bloods sample, a single dose of 20 mg/kg BH_4 (sapropterin dihydrochloride, KUVAN®, Merck Serono) was administered orally and samples were collected at 8 h (T2) and 24 h (T3) after BH_4 ingestion. Time points T0 and T1 of Tests 1 and 2 were, therefore, equivalent to each other, whereas T2 and T3 were differed in that BH_4 administration was included in Test 2.

L-Phe and BH_4 were dissolved in orange juice before administration. Patients were told to fast for at least 1 h before all blood collections.

Phe and Tyr plasma concentrations were measured using tandem mass spectrometry (MS/MS) in the Laboratory of Inborn Errors of Metabolism of SGM/HCPA, as described by Rashed et al. [25]. All measurements were made in duplicate, and the mean of the two measurements was calculated. In Test 2 samples, the levels of BH_4 (total biopterins) were also measured, according to the method of Opladen et al. [26].

2.3. Responsiveness to BH_4

Patients were defined as responsive to BH_4 if they met at least one of the criteria listed below:

Criterion A: this criterion used Phe values at T1 [3 h after Phe loading in Tests 1 (1T1) and 2 (2T1)] and at T2 [11 h after Phe loading in Tests 1 (1T2) and 2 (2T2) and 8 h after BH_4 administration in Test 2]. The following equation was used for calculations: $[((2T2-2T1)/2T1)\times 100]-[((1T2-1T1)/1T1)\times 100]$.
Individuals were responsive if the values found corresponded to a reduction of ≥30% in Phe levels in Test 2.
Criterion B: this criterion used Phe values at T1 and T3 [27 h after Phe loading in Tests 1 (1T3) and 2 (2T3) and 24 h after BH_4 administration in Test 2]. The following equation was used for calculations: $[((2T3-2T1)/2T1)\times 100]-[((1T3-1T1)/1T1)\times 100]$.
Individuals were responsive if the values found corresponded to a reduction of ≥30% in Phe levels in Test 2.
Criterion C: this criterion used the percentage difference of the value found for the area under the Phe curve in Tests 1 (AUC1) and 2 (AUC2). The following equation was used for calculations: $[((AUC2-AUC1)/AUC1)\times 100]$. Individuals were responsive if the difference was ≥30%, as long as the Test 1 area was greater than the Test 2 area.

To compare the classification of responsiveness, four additional criteria were used, as described below:

Criterion D: only the Phe values at T1 and T2 of Test 2 were used. The following equation was used for calculations: $[((2T2-2T1)/2T1)\times 100]$. Individuals were responsive if the values found corresponded to a reduction of ≥30% in Phe levels in time point 2.
Criterion E: only the Phe values at T1 and 3 of Test 2 were used. The following equation was used for calculations: $[((2T3-2T1)/2T1)\times 100]$. Individuals were responsive if the values found corresponded to a reduction of ≥30% in Phe levels in time point 3.
Criterion F: this criterion was used by the authors in a previous study [23] to evaluate the responsiveness of 5 patients also included in this study, and was defined as a reduction of ≥30% in Phe levels 8 h after simple BH_4 loading (single-dose of BH_4 at 20 mg/kg, without a concomitant load of Phe or L-Phe).

Criterion G: this criterion was used by the authors in a previous study [23] to evaluate the responsiveness of 5 patients also included in this study, and was defined as a reduction of ≥30% in Phe levels 24 h after simple BH_4 loading.

2.4. Dietary intake of Phe

Phe intake was evaluated using food recalls on the day before and on the first day of Tests 1 and 2, which totaled, therefore, two recalls for each Test. Dietary Phe intake was calculated using the nutrition-support software NutriBase (NB7), Clinical Edition. All patients received instructions to keep the same dietary Phe prescription that they followed before the beginning of the study.

2.5. Genotype

Genotypes of patients 1 to 17 (Table 1) were established previously and retrieved from their medical charts. Patient 18 was the only patient who had not been genotyped at the time of the study.

2.6. Statistical analysis

The Statistical Package for Social Sciences 18.0 (SPSS® Inc., Chicago, IL) was used for statistical analysis. Data were described using absolute and relative frequencies. The Stata program was used to calculate the area under the Phe curve. Continuous variables were expressed as mean ± standard deviation or median and interquartile range. The Shapiro–Wilk test was used to analyze variable distribution.

The Student *t* test was used to compare Phe intake between the different food recalls, the area under the Phe curve, and the difference in Phe levels between the collection time points for responsive patients versus non-responsive patients. Phe plasma concentrations and Phe:Tyr ratios were analyzed using Generalized Estimating Equations and Bonferroni correction. The comparison between BH_4 responsiveness criteria was made using kappa statistics.

The level of significance was set at 5%.

3. Results

Eighteen patients (11 girls and 7 boys) from 16 nonrelated families were included in the study. Parental consanguinity was found in 1/16 (6.25%) family. Median patient age was 12 (interquartile range: 10–16.5) years; minimum age was 6, and maximum, 31 years.

Mean Phe levels in Tests 1 and 2 at T0 were 255 ± 138 and 333 ± 173 μmol/L ($n = 18$; $p = 0.342$), and at T1, 730 ± 221 and 790 ± 310 ($n = 18$; $p = 1.0$). In both Tests 1 and 2 there were significant increases in Phe levels between T0 and T1 ($p < 0.01$). There were no statistically significant differences in Phe:Tyr ratios when the same time points were compared between Tests 1 and 2.

BH_4 plasma levels in Test 2 increased after L-Phe administration (T1) and BH_4 loading (T2) (data not reported).

3.1. Response to BH_4

Six patients (33.3%) were responsive, four according to criterion A, three according to criterion B, and four according to criterion C (Table 2). The comparison of BH4-responsiveness according to criterion used is shown in Table 3. In the calculation of the Kappa Index, the data reported by Giugliani et al. [23] were not included because of the small sample size (5/18 patients included in this study). Kappa was 0.557 when criteria A + B were compared with criterion C ($p = 0.017$). For the other criteria, Kappa values indicated a weak agreement.

For responsive patients, mean Phe level variations between time points T1 and T2 and between time points T1 and T3 were different only in Test 2 ($p = 0.002$ and 0.011, respectively), that difference was not found for non-responsive patients (Table 2). The analysis of area under the curve (AUC) revealed a statistically significant difference between Tests 1 and 2 mean values for responsive patients ($p < 0.01$), but not for non-responsive patients ($p = 0.242$) (Table 2).

Fig. 1 shows the Phe plasma concentration values found in this study according to collection time point in Tests 1 and 2 for a BH_4-responsive patient (Fig. 1A) and a non-responsive patient (Fig. 1B).

Table 1
Genotype, PKU phenotype for each patient and comparison of responsiveness in this study with findings in the literature.

Patient	Type of PKU	Genotype		Responsiveness to BH_4[a] (our study)	Responsiveness to BH_4 (number of patients included)[b]	Protocol used in the literature[c]
		Allele 1	Allele 2			
1	Classical	p.I65T	p.R408W	R	R (2)	BH_4(10 mg) − 24 h
2	Classical	p.V388M	p.V388M	R	R (1) − Slow R (1)[d]	BH_4(20 mg) − 24 h
3	Classical	p.I65T	p.R176X	NR	Nd	Nd
4	Classical	p.R252W	p.R261Q	NR	Nd	Nd
5	Classical	p.165T	p.I65T	NR	NR (1)	Phe(100 mg)/BH_4(20 mg) − 8 h
6	Mild	p.E390G	p.R408Q	R	Nd	Nd
7	Classical	p.R261X	p.R176X	NR	Nd	Nd
8	Mild	p.R408W	c.165delT	NR	Nd	Nd
9	Mild	p.R408W	c.165delT	NR	Nd	Nd
10	Mild	p.E390G	p.A104D	R	Nd	Nd
11	Mild	p.R408W	p.L348V	NR	Nd	Nd
12	Mild	p.R408W	p.L348V	NR	Nd	Nd
13	Mild	p.A300S	p.L311P	R	Nd	Nd
14	Classical	p.R408W	p.R408W	NR	NR(13) Slow R (1)[d]	BH_4(20 mg) − 24 h (13)/Other (1)
15	Mild	p.R261Q	p.V388M	R	R (1)	BH_4(10 mg) − 8 h
16	Mild	p.L249F	p.V388M	NR	NR (1)	BH_4(20 mg) − 24 h
17	Classical	c.165delT	?	NR	–	–
18	Mild	?	?	NR	–	–

PKU: phenylketonuria; Nd: not described; R: Responsive; NR: non-responsive; Siblings: 8 and 9; 11 and 12.
[a] To be classified as responsive, patient had to obtain a positive result according to at least one of the criteria used (criteria A, B and C).
[b] Data retrieved from BIOPKUdb http://www.bh4.org/BH4DatabasesBioHPA-PAH.asp.
[c] Loading protocol used in other PKU patients with the same genotype (amount of BH_4 per kg of current weight; time used for responsiveness criterion).
[d] Patients with a Phe reduction of 20 to 30% after BH_4 loading.

Table 2
BH_4 responsiveness: Phe values and corresponding areas under the curve (n = 18 patients).

Patients	Test 1			Test 2			Area under Phe curve		Criterion A (%)	Criterion B (%)	Critérion C (%)
	Phe (μmol/L)			Phe (μmol/L)			AUC1	AUC2			
	T1	T2	T3	T1	T2	T3					
Responsive											
1	852	1032	949	1010	823	816	373.1	289.8	−39.6	−30.6	−22.3
2	1193	1314	849	939	895	639	435.2	297.2	−14.8	−3.1	−31.7
6	588	474	136	570	144	74,8	133.8	27.9	−55.3	−10	−79.1
10	522	372	113	612	127	58,4	103.1	19.3	−50.5	−12.1	−81.3
13	744	602	550	748	516	189	207.1	145.2	−11.9	−48.6	−29.9
15	657	822	589	819	498	355	281.6	153.1	−64.3	−46.3	−45.6
μ ± sd	759 ± 242	769 ± 358	531 ± 349	783 ± 175*	500 ± 324*	355 ± 312*	255.6 ± 132.0**	155.4 ± 120.9**	−39.4 ± 21.7	−25.1 ± 19.5	−48.3 ± 25.8
Non-responsive											
3	1095	750	706	991	946	808	253.2	327.3	27	17	29.3
4	702	555	462	496	553	390	185.9	186.6	32.4	12.8	0.4
5	517	427	530	652	661	494	161.1	223.4	18.7	−26.7	38.6
7	816	775	617	634	696	542	263.5	239.5	14.8	9.9	−9.1
8	367	425	390	699	726	715	152.9	263.0	−11.9	−3.9	72.0
9	544	626	486	742	736	730	216.4	265.8	−15.8	9	22.8
11	836	655	613	608	513	418	225.7	172.6	6	−4.5	−23.5
12	991	925	717	1095	855	660	311.9	280.7	−15.2	−12	−10.0
14	711	562	296	587	421	197	171.3	123.6	−7.3	−8	−27.8
16	789	640	446	714	637	422	206.6	206.4	8.1	2.5	−0.1
17	790	810	799	1810	1787	1398	293.2	608.1	−3.8	−23.9	107.4
18	422	350	227	489	304	208	111.7	93.1	−20.8	−11.2	−16.7
μ ± sd	715 ± 220	625 ± 172	524 ± 174	793 ± 367	736 ± 375	582 ± 323	212.8 ± 60.0	249.2 ± 131.0	2.6 ± 17.8	−3.2 ± 14	15.3 ± 41.1

Phe: phenylalanine; Test 1: simple Phe loading; Test 2: combined Phe + BH_4 loading; Siblings: 8 and 9; 11 and 12.
Criterion A: [((2T2 − 2T1)/2T1) × 100] − [((1T2 − 1T1)/1T1) × 100].
Criterion B: [((2T3 − 2T1)/2T1) × 100] − [((1T3 − 1T1)/1T1) × 100].
Criterion C: [((AAC2 − AAC1)/AAC1) × 100].
To be classified as responsive, patient had to obtain a positive result according to at least one of the criteria used (criteria A, B and C).
T1 — time point 1: Phe level 3 h after Phe loading; T2 — time point 2: Phe level 11 h after Phe loading and 8 h after BH_4 loading (Test 2); T3 — time point 3: Phe level 27 h after Phe loading and 24 h after BH_4 loading (Test 2); AUC1: Area under the curve in Test 1; AUC2: Area under the curve in Test 2.
* Phe variation between T2 and T1 and between T3 and T1 was statistically significant (p = 0.002 and 0.011).
** Statistically significant difference (p < 0.01).

3.2. Association between genotype and responsiveness to BH_4 (Table 1)

Data about the genotype, severity of PKU and BH4-responsiveness are shown in Table 1.

3.3. Dietary intake

The patients ingested in average 705.56 ± 356.77 and 608.89 ± 405.51 mg (mean ± SD) of Phe per day, according to dietary recalls 1 and 2 of Test 1 (p = 0.446), and 672.78 ± 406.17 and 572.78 ± 329.47 mg (mean ± SD) of Phe per day according to dietary recalls 1 and 2 of Test 2 (p = 0.281). There were no statistically significant differences in Phe intake between Tests 1 (657.2 ± 379.6 mg Phe/day) and 2 (622.7 ± 368 mg Phe/day) (p = 0.564).

4. Discussion

In this study, patients with PKU that might potentially benefit from the use of BH_4 were identified by comparing their Phe levels after a simple Phe loading test and after a combined Phe + BH_4 loading test. Although no consensus has been reached in the literature about the most adequate method to identify these individuals, Phe plasma levels should be elevated at the time of BH_4 administration to induce an increase in PAH activity and, consequently, to potentiate the effect of BH_4 [27]. This recommendation adds complexity to the investigation of BH_4 responsiveness in patients with good adherence to dietary treatment. The increase of Phe levels is often achieved by increasing the ingestion of dietary Phe [8,9,20,22,28–30], which may raise ethical and psychological issues. A single Phe dose using L-Phe may also be administered, with the advantage that the patient's diet does not have to be changed [19,31–33]; however, Phe levels after this type of loading tend to spontaneously decrease in 24 h even when BH_4 is not been administered [20,32]. Therefore, BH_4 responsiveness would have to be confirmed by comparing Phe plasma levels after the Phe + BH_4 loading test and after the simple Phe loading test. We have found in the literature only two studies performing the simple Phe loading test and the combined Phe + BH_4 loading test in the

Table 3
BH_4 responsiveness according to criteria used for classification in this study.

Patients	Criterion A	Criterion B	Criterion C	Criterion D	Criterion E	Criterion F[a]	Criterion G[a]
1	R	R	NR	NR	NR	NR	R
2	NR	NR	R	NR	R	–	–
3	NR	NR	NR	NR	NR	R	R
4	NR	NR	NR	NR	NR	–	–
5	NR	NR	NR	NR	NR	–	–
6	R	NR	R	R	R	–	–
7	NR	NR	NR	NR	NR	–	–
8	NR	NR	NR	NR	NR	–	–
9	NR	NR	NR	NR	NR	–	–
10	R	NR	R	R	R	–	–
11	NR	NR	NR	NR	R	R	R
12	NR	NR	NR	NR	R	NR	NR
13	NR	R	NR	R	R	–	–
14	NR	NR	NR	NR	R	–	–
15	R	R	R	R	R	–	–
16	NR	NR	NR	NR	R	NR	NR
17	NR	NR	NR	NR	NR	–	–
18	NR	NR	NR	R	R	–	–

R = responsive; NR = non-responsive.
The criteria are defined in the Material and methods section of this study.
Siblings: 8 and 9; 11 and 12.
[a] Data reported by Giugliani et al. [23].

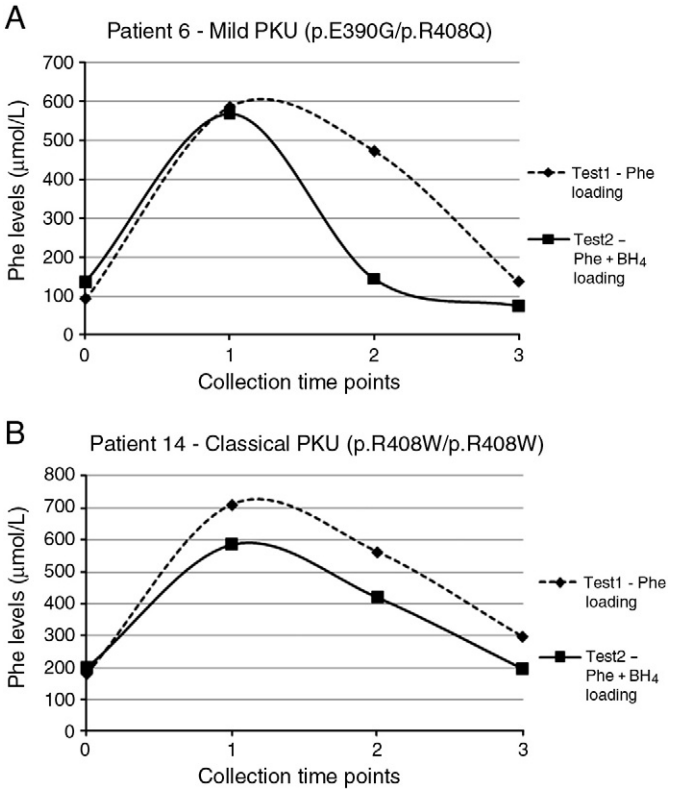

Fig. 1. (A) Phe variation in a patient classified as responsive to BH_4 according to collection time points. (B) Phe variation in a patient classified as non-responsive to BH_4 according to collection time points.

same patient. The one conducted by Desviat et al. [20] included six patients with Phe level at diagnosis below 360 µmol/L, but classified their responsiveness only according to the combined Phe + BH_4 test; and the one conducted by Ponzone et al. [32], which included seven patients with different forms of PKU, but did not establish clear criteria for the classification of responsiveness. The analysis of Phe curves in both studies suggests that Phe plasma levels reach similar values at the end of the simple Phe and combined Phe + BH_4 loading tests; although, Phe levels seem to decrease faster when BH_4 is used.

The protocol to evaluate responsiveness in this study is innovative for two reasons: (1) it included the use of a simple Phe and a combined Phe + BH_4 loading test for 18 patients with PKU and good metabolic control; (2) it defined BH_4 responsiveness parameters based on the comparison of results of these two tests. According to the strategy adopted in this study, 33.3% (6/18) of the patients were classified as responsive, which is in agreement with data in the literature [17–19,23,30,34]. Of the responsive patients, two had the classical PKU (n = 2/8; 25.5%) and four, the mild PKU (n = 4/10; 40%), which confirmed that responsiveness is greater among individuals with milder forms of PKU. Moreover, of the 16 patients for whom genotypes were available, six had data about BH4-responsiveness genotypes described in the literature.

There was also an important discordance between the seven criteria used to compare the definition of responsiveness. In the group of 18 individuals, only one was responsive and six were non-responsive according to all criteria. If criteria 4 and 5, which consider the results of combined Phe + BH_4 loading only, had not been used, a greater number of patients would have concordant results for non-responsiveness (n = 10). The use of criteria 4 and 5 independently seems to lead to a greater number of diagnoses of patients responsive to BH_4; this may be explained by the fact that these criteria do not take into consideration that Phe may be excreted spontaneously and not due to BH_4 action.

Although the protocol here described has some limitations, e.g. the need for patients to attend the treatment center twice (two consecutive weeks) and to undergo a higher number of blood collections, the superiority of this protocol when compared with others already described in the literature can only be assessed if comparative studies are carried out. This underscores the need for determining the sensitivity, specificity, as well as positive and negative predictive values of these tests, especially if we take into consideration that, as our findings clearly show, patients diagnosed as BH_4-responsive according to one given protocol may not be diagnosed as such in another protocol. In this sense, one of the future goals of the authors is to test simple BH_4 loading in all patients included in the present study.

4.1. Comments on dietary ingestion and Phe levels during the Tests

Dietary Phe ingestion did not change in the two recalls in the same Test, and there was also no variation in the comparison of total Phe amount ingested in Test 1 and in Test 2, which suggests that the fall in Phe levels in responsive individuals was secondary to BH_4 administration.

The variation of Phe plasma concentrations along the study revealed an elevation at time point T1 in comparison with time point T0, that is, 3 h after Phe loading, which demonstrates, therefore, that L-Phe was absorbed by the patient. The collection point 3 h after Phe loading was used because it has been described in the literature as the point at which Phe plasma levels peak predominantly [24]. However, in 9 patients in this study, the highest Phe plasma concentration values occurred at least, in one of the Tests, 11 h after Phe loading, which has also been reported by other studies [19,20].

Phe plasma concentrations at time points 0 and 1 in the two Tests did not differ from each other, which emphasizes their comparability.

4.2. Conclusions

BH_4 has emerged as a new treatment for patients with mostly milder forms of PKU and may substantially improve their quality of life. Numerous positive findings, including increases in Phe tolerance, have been reported in association with the use of this medication. However, no consensus has been reached about the best method and criteria to define responsiveness to BH_4. The validation of methods and criteria for this purpose is fundamental to optimize the treatment with BH_4 also in terms of cost and effectiveness. Our data suggest that, in responsive individuals, Phe levels decrease faster after Phe + BH_4 loading than after simple Phe loading, and confirm that the comparison between simple Phe and combined Phe + BH_4 loading is valid to evaluate responsiveness. Moreover, because of the wide range of variation of responsiveness classification for each patient, more than one criterion should be used to establish a definition, and these criteria should take into consideration the comparison between the values obtained in single Phe and combined Phe + BH_4 loading tests.

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